Gel Filtration of Sialoglycoproteins

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The role of sialic acid in the gel-filtration behaviour of sialoglycoproteins was investigated by using the separated isoenzymes of purified human liver α -L-fucosidase and several other well-known sialic acid-containing glycoproteins (fetuin, α_1 -acid glycoprotein, thyroglobulin and bovine submaxillary mucin). For each glycoprotein studied, gel filtration of its desialylated derivative gave an apparent molecular weight much less than that expected just from removal of sialic acid. For the lower-molecular-weight glycoproteins (fetuin and α_1 -acid glycoprotein), gel filtration of the sialylated molecules led to apparent molecular weights much larger than the known values. The data indicate that gel filtration cannot be used for accurately determining the molecular weights of at least some sialoglycoproteins.

Gel filtration is widely used to determine approximate molecular weights of macromolecules. It has been shown that glycoproteins give anomalous results on gel filtration (Andrews, 1965), particularly carbohydrate-rich glycoproteins (Spiro, 1973). Since more and more proteins are being shown to be glycoproteins (Spiro, 1973), it seemed important to investigate the role of the carbohydrate moieties in causing the anomalous gel-filtration behaviour of glycoproteins.

Human liver α -L-fucosidase (EC 3.2.1.51) has been shown to be a sialoglycoprotein (Alhadeff & Freeze, 1977) and there is considerable evidence that the more acidic isoenzymes of α -L-fucosidase are chemically related to the more neutral forms by sialic acid residues (Alhadeff *et al.*, 1974, 1975a; Turner *et al.*, 1974, 1975; Thorpe & Robinson, 1975; DiMatteo *et al.*, 1976). In the present study I have investigated the role of sialic acid on the gel-filtration behaviour of human liver α -L-fucosidase (and its isoenzymes) and on several other well-known sialoglycoproteins (fetuin, thyroglobulin, α_1 -acid glycoprotein and bovine submaxillary mucin).

Experimental

Materials

Neuraminidase (*Clostridium perfringens*, type VI) and neuraminidase coupled to agarose (Sephacrose) were purchased from Sigma (St. Louis, MO, U.S.A.). Ampholytes for isoelectric focusing were purchased from LKB Produkter (Pleasant Hill, CA, U.S.A.). The Sepharose 6B was purchased from Pharmacia (Piscataway, NJ, U.S.A.). The purified human liver α -L-fucosidase used was a sample from a previous

affinity-chromatographic purification, which has been described in detail elsewhere (Alhadeff & Freeze, 1977).

Methods

All procedures were carried out at 0–4°C unless otherwise stated. α -L-Fucosidase activity was assayed with *p*-nitrophenyl α -L-fucopyranoside (Sigma) as previously described (Alhadeff *et al.*, 1974). Absorbances were read at 420 nm on a Gilford 2400-S spectrophotometer. A unit of α -L-fucosidase is the amount of enzyme necessary to hydrolyse 1 nmol of substrate/min at 37°C. Enzyme solutions were concentrated by ultrafiltration by using Amicon concentrators with UM-10 Diaflo membranes at 345–483 kPa (50–70 lb/in²). Protein was determined by the method of Lowry *et al.* (1951), with human serum albumin as standard.

Isoelectric focusing. Preparative isoelectric focusing of purified α -L-fucosidase (3200 units) was performed with a 110 ml column (LKB) in a gradient of 0–67% (w/v) sucrose containing 1% ampholytes (pH 5–8) as previously described (Alhadeff *et al.*, 1975a). The temperature was maintained at 4–6°C with a circulating-water bath (Brinkmann Lauda K-2/R). Electrofocusing was conducted for 65 h starting at 2.0 mA and 450 V, after which fractions (approx. 0.7 ml) were collected in tubes and corked. The pH of each fraction was measured at 0–2°C with a Beckman Digital pH-meter, and 50 μ l portions of each fraction were assayed for 60 min for α -L-fucosidase activity.

Starch-gel electrophoresis. Vertical starch gels were run by the method of Smithies (1959) on 10–30 μ l samples of separately combined, dialysed [against ice-cold 10 mM-NaH₂PO₄, pH 5.5 (0.02%, w/v),

NaN_3]] and concentrated α -L-fucosidase [peaks I–VII from preparative isoelectric focusing at pH 5.9; gel buffer was 5 mM-citric acid/sodium phosphate buffer, pH 5.8; discontinuous bridge buffer was 40 mM-citric acid/sodium phosphate buffer, pH 5.9, and 10% (w/v) NaCl] for 3 h at 25 mA and 300 V. The gels were incubated for 45 min at 37°C with 1.0 mM-4-methylumbelliferyl α -L-fucopyranoside (Koch–Light, Colnbrook, Bucks., U.K.) and the bands of fucosidase activity were detected with a u.v. lamp after exposure of the gels to glycine/sodium carbonate, pH 10.0.

Desialylation of glycoproteins. Asialofetuin was prepared by hydrolysing 1.0 g of fetuin (from foetal calf serum; Sigma) in 400 ml of 12.5 mM- H_2SO_4 at 80°C for 1 h (Spiro, 1960) and dialysing extensively against water, followed by freeze-drying.

Asialo-(α_1 -acid glycoprotein) was prepared by hydrolysing 1.0 g of human α_1 -acid glycoprotein (generously provided by the American Red Cross National Fractionation Center, Bethesda, MD, with the partial support of NIH grant no. HL 13881) in 400 ml of 12.5 mM- H_2SO_4 at 80°C for 1 h (Morell *et al.*, 1971). The hydrolysate was filtered, dialysed extensively against water and freeze-dried.

Asialo-(bovine submaxillary mucin) was prepared by hydrolysing 60 mg of bovine submaxillary mucin (Sigma) in 80 ml of 0.01 M-HCl at 80°C for 100 min (Tettamanti & Pigman, 1968) followed by extensive dialysis against water, centrifugation at 5000g for 8 min and freeze-drying of the supernatant.

Asialo-thyroglobulin was prepared by incubating 20 mg of bovine thyroglobulin (Sigma, type 1) with 1 unit (a unit liberates 1.0 μ mol of sialic acid/min with sialyl-lactose as substrate) of *Cl. perfringens* neuraminidase attached to beaded agarose (Sigma) for 5 h at 37°C in 2.0 ml of 0.1 M-citric acid/sodium citrate buffer, pH 5.0. After incubation the mixture was centrifuged at 22000g for 15 min to separate the insoluble agarose-neuraminidase from the soluble asialo-thyroglobulin.

The Warren (1959) thiobarbituric acid assay for sialic acid was performed on the sialoglycoproteins and their desialylated counterparts to monitor the extent of desialylation.

Human liver α -L-fucosidase (108 units) was desialylated by incubation with 5 units of neuraminidase (*Cl. perfringens*, type VI; Sigma) for 27 h at 37°C. The neuraminidase contained less than 0.012 unit of proteinase activity/mg of protein with casein as substrate. A unit of proteinase will hydrolyse casein to produce colour equivalent to 1.0 μ mol (181 μ g) of tyrosine/min at pH 7.5 at 37°C with Folin–Ciocalteu Phenol Reagent (Sigma).

Gel filtration. Samples of liver α -L-fucosidase and its isoenzymes (20–160 units) as well as the sialoglycoproteins (3.3 mg) and their desialylated counterparts (2.0–5.0 mg) were subjected to gel filtration on a

column (0.9 cm $\times$ 102 cm) of Sepharose 6B, which fractionates between 25000 and 2500000 mol.wt. (Pharmacia). The column was calibrated with 3.3 mg of the following standard proteins: human urinary β -galactosidase (supplied by Dr. B. Kress; University of California, San Diego; mol.wt. 570000); aldolase (Pharmacia; mol.wt. 158000); bovine serum albumin (Sigma; mol.wt. 67000); ovalbumin (Pharmacia; mol.wt. 45000); α -chymotrypsinogen A (Sigma; mol.wt. 25000); bovine pancreatic ribonuclease A (Pharmacia; mol.wt. 13700). All of the standard proteins, except for β -galactosidase, are known to contain no sialic acid (Sober, 1970). The carbohydrate composition of β -galactosidase has not been published. These proteins were applied in 300 μ l of 10 mM- NaH_2PO_4 , pH 5.5, containing 0.02% (w/v) NaN_3 and eluted with this same buffer. The elution volumes were determined by multiplying the average volume per fraction by the fraction number of maximal A_{280} (for standard proteins) or maximal enzymic activity (for α -L-fucosidase and its isoenzymes). The void volume of the column was determined with Blue Dextran to be 26.7 ml. The results of gel filtration were plotted as logarithm of molecular weight versus K_{av} , where $K_{av} = (V_e - V_0)/(V_t - V_0)$, V_e is elution volume of protein, V_0 is void volume (26.7 ml) and V_t is bed volume (65.0 ml).

Results

Preparative isoelectric focusing was performed on purified liver α -L-fucosidase to separate the seven isoelectric forms between pH 5.0 and 6.9 (Alhadeff *et al.*, 1975a). The seven forms were each combined separately, dialysed and concentrated. To determine whether the forms had been adequately separated by isoelectric focusing, starch-gel electrophoresis was performed at pH 5.9 on each of the seven forms. At this pH, forms I–III should migrate towards the cathode and forms IV–VII towards the anode. Fig. 1 indicates that each isoelectric-focusing peak contained predominantly one form of α -L-fucosidase with the expected electrophoretic migration. These forms were subjected to gel filtration on Sepharose 6B. When the results of gel filtration for several standard proteins were plotted as the logarithm of the molecular weight versus K_{av} , the curve approximated to a straight line. This standard curve was used for determining the apparent molecular weights of the sialoglycoproteins subjected to gel filtration. Fig. 2 represents the elution profile of the five isoenzymes (III–VII) of liver α -L-fucosidase, which are considered to be related chemically, at least in part, by sialic acid residues (Alhadeff *et al.*, 1975a). Fig. 2 shows that the elution volume (and hence the apparent molecular weight) for these five isoenzymes appears to be related to their isoelectric points. The form with the most acidic pI (VII) has the smallest elution

Fig. 1. Starch-gel electrophoresis of human liver α -L-fucosidase separated by preparative isoelectric focusing. See the Materials and Methods section for details.

Fig. 2. Elution profile of α -L-fucosidase isoenzymes III–VII from a column (0.9 cm $\times$ 102 cm) of Sepharose 6B. See the Materials and Methods section for details.

volume and the form with the most neutral pI (III) has the largest elution volume. For all five isoenzymes, the larger the pI of the form the larger the elution volume. Varying the amount of activity put on the gel-filtration column for a particular isoenzyme did not affect its elution volume. Table 1 summarizes the gel-filtration data on the isoenzymes of α -L-fucosidase. Isoenzymes I and II do not fit the pattern of the other five isoenzymes, but these two forms are probably not sialylated (Alhadeff *et al.*, 1975a). Also included in Table 1 are the data for the whole unseparated α -L-fucosidase and its neuraminidase-treated derivative. Unlike a previous study, in which one high-molecular-weight form (175000) was found on Sepharose 4B chromatography (Alhadeff *et al.*, 1975b), two lower-molecular-weight forms (A and B) were found for normal α -L-fucosidase (76000 and 18000). After neuraminidase treatment, the two forms were eluted with the considerably lower apparent mol.wts. of 43000 and below 10000, the latter value being off-scale with the column used in the present study. The exact relationship of the two gel-filtration forms of α -L-fucosidase (A and B) to the seven α -L-fucosidase isoenzymes is not presently known. Full α -L-fucosidase activity was recovered after neuraminidase treatment, suggesting that the lower molecular weights after treatment were not due to degradation.

Several other well-known sialoglycoproteins and their desialylated derivatives were subjected to gel filtration on the Sepharose 6B column. Assay of the desialylated glycoproteins for sialic acid by the Warren (1959) thiobarbituric acid assay indicated that 71–95% of the sialic acid had been removed from the glycoproteins. For thyroglobulin 50% of the sialic acid was removed by treatment with agarose-neuraminidase. Table 2 summarizes the

Table 1. Gel-filtration behaviour of human liver α -L-fucosidase and its isoenzymes on Sepharose 6B. The symbols and the data in this Table are discussed in the text.

Sample	Elution volume (ml)	K_{av}	Calculated mol.wt.
Purified liver α -L-fucosidase			
Form A	55.3	0.747	76000
Form B	64.8	0.995	18000
Neuraminidase-treated purified liver α -L-fucosidase			
Form A	59.1	0.846	43000
Form B	69.5	1.117	<10000
Isoenzyme I (pI 6.9)	57.2	0.796	57000
Isoenzyme II (pI 6.7)	64.8	0.995	18000
Isoenzyme III (pI 6.3)	67.4	1.063	$\approx$ 12000
Isoenzyme IV (pI 5.9)	63.8	0.969	21000
Isoenzyme V (pI 5.6)	55.3	0.747	76000
Isoenzyme VI (pI 5.4)	51.4	0.645	137000
Isoenzyme VII (pI 5.0)	49.5	0.595	185000

Table 2. *Gel-filtration behaviour of sialoglycoproteins and their desialylated derivatives on Sepharose 6B*
Molecular weights and sialic acid contents of glycoproteins are taken from Sober (1970).

Glycoprotein	Sialic acid content (g of sialic acid/100 g of glycoprotein)	Elution volume (ml)	K_{av}	Mol.wt.	
				Calculated	Known
Fetuin	8.8	45.3	0.486	350 000	48 000
		44.6	0.468	385 000	
				Av. 368 000	
Asialofetuin	0.6†	68.0	1.056	12 500	44 000
		68.2	1.083	10 500	
				Av. 11 500	
α_1 -Acid glycoprotein	12.1	44.6	0.467	386 000	44 000
		44.6	0.467	386 000	
				Av. 386 000	
Asialo-(α_1 -acid glycoprotein)	0.6†	49.0	0.582	197 000	39 000
		49.4	0.593	185 000	
				Av. 191 000	
Thyroglobulin	1.4	32.0	0.137	>570 000	669 000
		33.3	0.172	>570 000	
				Av. >570 000	
Asialo-thyroglobulin	0.7†	61.6	0.910	29 300	660 000
		59.8	0.864	38 000	
				Av. 34 000	
Bovine submaxillary mucin	2.5	27.6*	0.022	>570 000	1 300 000
		26.5*	0.006	>570 000	
				Av. >570 000	
Asialo-(bovine submaxillary mucin)	0.7†	59.4	0.854	40 500	1 267 000
		62.3	0.930	26 000	
				Av. 33 000	

* These values are very close to the void volume (26.7 ml).

† These values were determined by the Warren (1959) method after desialylation of the glycoproteins by either acid hydrolysis or treatment with neuraminidase (see the Experimental section).

gel-filtration behaviour data for these sialoglycoproteins and their desialylated derivatives. The values are given for two gel-filtration runs for each glycoprotein to show the variability for the same glycoprotein. The apparent mol.wts. for fetuin (368 000) and α_1 -acid glycoprotein (386 000) are much greater than their known mol.wts. (48 000 and 44 000 respectively) (Spiro, 1973). Thyroglobulin had an elution volume smaller than that of any of the standard proteins and therefore its mol.wt. can only be estimated as over 570 000. For bovine submaxillary mucin the elution volume (27 ml) was very near the void volume (26.7 ml) of the column, and these results indicate only that bovine submaxillary mucin is a very large molecule. For all the glycoproteins studied, except α_1 -acid glycoprotein, gel filtration of the desialylated glycoproteins gave apparent molecular weights that were a small percentage (3–26%) of their known value. For asialo-(α_1 -acid glycoprotein), the apparent molecular weight was about half of that for the sialylated compound, but about five times larger than the known weight for α_1 -acid glycoprotein. For each glycoprotein, the apparent molecular weight

is considerably larger (2–32-fold) than that of its desialylated derivative.

Discussion

In the present investigation the role of sialic acid on the gel-filtration behaviour of sialoglycoproteins has been studied. Purified human liver α -L-fucosidase and its five most acidic isoenzymes (III–VII), which are thought to be chemically related by sialic acid residues, were used as model glycoproteins for these studies. The data on α -L-fucosidase and its isoenzymes suggest that the presence of sialic acid residues leads to smaller gel-filtration elution volumes (and higher apparent molecular weights). A similar finding has been found for human serum α -L-fucosidase and its desialylated derivative prepared by incubation with proteinase-free neuraminidase (Alhadeff & Janowsky, 1978). In this case the normal enzyme had an apparent mol.wt. of 296 000 and the apparent mol.wt. of its desialylated derivative was greatly decreased to 37 000. To determine whether the data on α -L-fucosidase were unique or had more general applic-

ability, several other well-known sialoglycoproteins and their desialylated derivatives were subjected to gel filtration on the Sepharose 6B column. For each glycoprotein studied, the apparent molecular weight of the desialylated compound was smaller than that of the untreated glycoprotein by an amount considerably greater than that caused by the small weight loss due to removal of sialic acid. For the lower-molecular-weight glycoproteins (fetuin and α_1 -acid glycoprotein), the apparent molecular weights of the sialylated compounds obtained by gel filtration were 8–9 times their known molecular weights. Except for α_1 -acid glycoprotein, the desialylated glycoproteins gave apparent molecular weights considerably lower than their known values. A recent study has shown that the presence of sialic acid in peptidyl-oligosaccharides causes anomalously high molecular weights as determined by gel filtration on Bio-Gel P-4 and P-6 (Etchison *et al.*, 1977). In their study compounds containing sialic acid were excluded from the gel-filtration beads more than neutral oligosaccharides of similar molecular weight.

At present it is not clear what causes the large difference in gel-filtration behaviour between sialoglycoproteins and their desialylated derivatives. It is possible that the presence of sialic acid in molecules leads to aggregation and the formation of multimers. In this regard, it has been suggested that the larger of the two forms of liver α -L-fucosidase found by gel filtration on Sephadex G-200 might be an aggregate of the lower-molecular-weight form (Thorpe & Robinson, 1975). It is also possible that the presence of sialic acid in certain molecules leads to changes in tertiary structure (e.g. increase in molecular radii), which could account for their anomalous elution behaviour. Still another possibility is that the negative charge on sialic acid might somehow prevent molecules containing it from easily entering the interstices of gel-filtration beads. This phenomenon has been described as a charge-exclusion effect (Etchison *et al.*, 1977). Whatever the cause, it is important to know the empirical finding that the presence of sialic acid in a molecule drastically affects its gel-filtration behaviour and that the

molecular weights of many sialic acid-containing compounds could be inaccurately determined by this technique.

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